Activated Complement in Inflamed Aqueous Humor

Bartly J. Mondino,* M. Michael Glovsky,† and Ludwina Ghekiere†

Activated complement is an important mediator of inflammation. Radioimmunoassay was used to measure levels of C3a, an activated fragment of C3, in aqueous humor. Additionally, immunoelectrophoresis was performed on aqueous humor to detect Factor B and its conversion product, Bb, as well as C3c, a breakdown product of C3. All six samples of normal aqueous humor had no detectable C3a, C3c, or Factor B. All eight samples of aqueous humor from patients with anterior uveitis had measurably levels of C3a. Factor B and C3c were detected in 3/7 samples of inflamed aqueous humor. Factor B was converted fully to Bb in two of these three samples, suggesting alternative pathway activation of complement. Activated complement fragments are present in the aqueous humor of eyes with anterior uveitis and may help mediate the inflammatory process. Invest Ophthalmol Vis Sci 25:871-873, 1984

The complement system is not only a fundamental element of normal host defense against infection but also is involved in autoimmune tissue damage. The role of complement in anterior chamber inflammation is not known. Using hemolytic assays, Chandler and associates demonstrated C4 in samples of aqueous humor from all seven patients that they studied, but C3 and C1 were found only in samples from patients with anterior segment inflammation. Other studies showed that normal human aqueous humor contains functional C1, C4, C2, C3, C5, C6, and C7, but the small ratios of aqueous humor to serum measurements suggested that there was relatively little of these complement components in normal aqueous humor when compared with serum. The mean values of all these complement components and the median ratios of aqueous humor to serum measurements for each complement component were higher in inflamed than in normal aqueous humor. Moreover, Factor B, a component of the alternative pathway, was detected in inflamed but not normal aqueous humor.

Activated complement fragments are important mediators of inflammation with functions that include chemotaxis, anaphylatoxin activity, cytolsis, and immune adherence. Activation of C3, the pivotal component of the classical and alternative pathways, leads to cleavage of the molecule into two fragments: C3a (molecular weight 9,000) and C3b (molecular weight 171,000). Conversion of C3 is the hallmark event of complement activation. In this study, we measured levels of C3a by radioimmunoassay in human aqueous humor. The detection and quantitation of this cleavage product is a direct assessment of activated complement. Additionally, immunoelectrophoresis was performed on aqueous humor to detect Factor B and its conversion product, Bb, and C3c, a breakdown product of C3b.

Subjects and Methods. Samples of aqueous humor were obtained from 14 patients with the approval of the UCLA Human Subjects Protection Committee. Group 1 consisted of six patients undergoing routine cataract extraction. These patients had clear anterior chambers and were not receiving systemic or topical medications, except for 2% cyclopentolate hydrochloride and 2.5% phenylephrine hydrochloride, 2, 1, and ½ hr preoperatively.

Group 2 was composed of samples of aqueous humor from eight patients who had anterior uveitis (Table 1). Patients 1–5 underwent cataract extractions. Patients 6 and 8 had endophthalmitis and underwent anterior chamber and vitreous taps. Patient 7 underwent a penetrating keratoplasty for a perforated corneal ulcer that had been treated with butyl-2-cyanoacrylate tissue adhesive and a soft contact lens 4 days previously. The patients undergoing cataract extraction underwent preoperative dilation as described for patients in group 1. Patients 3 and 5 were treated with topical prednisolone acetate 1% topically prior to lens extraction.

Prior to collection of aqueous humor, all patients received lid and retrobulbar injections of 2% mepivacaine hydrochloride mixed with an equal volume of 0.75% bupivacaine hydrochloride. Approximately 0.15 ml of aqueous humor was collected from all patients in a 1-ml tuberculin syringe with a 30-gauge needle. The anterior chamber was entered through a superior limbal groove in the patients undergoing cataract extraction, through the trephine groove in the patient undergoing corneal transplantation and through the temporal limbus in the two patients with endophthalmitis. The aqueous samples were collected by one surgeon, were placed in ice immediately and picked up by a technician. The aqueous samples were stored at ~70°C.

Human C3a radioimmunoassay: Human C3a was purified to homogeneity as determined by SDS polyacrylamide gel electrophoresis employing a method described previously. Rabbit antibody to human C3a was obtained from Calbiochem-Behring (La Jolla, CA). C3a was labeled with 125I by the lactoperoxidase method as previously described.

Either cold C3a or C3aΔArg (C3a molecule minus the C-terminal arginyl residue that may be cleaved off...
Table 1. Aqueous humor from eyes with anterior uveitis

<table>
<thead>
<tr>
<th>Patient no./age (yrs.)</th>
<th>Diagnosis</th>
<th>Anterior chamber response</th>
<th>C3a (µg/ml)</th>
<th>Immunoelectrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/74</td>
<td>Cataract, proliferative diabetic retinopathy with vitreous hemorrhages and previous cryotherapy and photo-coagulation</td>
<td>Rare cell and trace flare</td>
<td>0.120</td>
<td>N.D.*</td>
</tr>
<tr>
<td>2/57</td>
<td>Cataract, recurrent iritis</td>
<td>Rare cell and trace flare</td>
<td>0.180</td>
<td>N.D.</td>
</tr>
<tr>
<td>3/19</td>
<td>Traumatic cataract with ruptured capsule</td>
<td>2+ cells and flare</td>
<td>0.133</td>
<td>N.D.</td>
</tr>
<tr>
<td>4/52</td>
<td>Intumescent lens, retinal detachment</td>
<td>2+ cells and flare</td>
<td>0.360</td>
<td>Factor B fully converted to Bb, C3c detected</td>
</tr>
<tr>
<td>5/40</td>
<td>Intumescent lens</td>
<td>2+ cells and flare</td>
<td>0.559</td>
<td>Factor B fully converted to Bb, C3c detected</td>
</tr>
<tr>
<td>6/35</td>
<td>Staphylococcus epidermidis corneal ulcer and endophthalmitis, proliferative diabetic retinopathy</td>
<td>4+ cells and flare, hypopyon</td>
<td>0.133</td>
<td>N.D.</td>
</tr>
<tr>
<td>7/63</td>
<td>Perforated herpes simplex corneal ulcer</td>
<td>4+ cells and flare</td>
<td>1.030</td>
<td>Insufficient sample</td>
</tr>
<tr>
<td>8/25</td>
<td>Traumatic endophthalmitis (Micrococcus isolated from vitreous)</td>
<td>4+ cells and flare, hypopyon</td>
<td>1.800</td>
<td>Factor B detected but not converted, C3c detected</td>
</tr>
</tbody>
</table>

* N.D. indicates that Factor B and C3c were not detected.

in vivo) was added in duplicate in concentrations of 1–80 ng/ml in a final volume of 1 ml to polystyrene tubes that were coated with rabbit anti-C3a. 5 125I-C3a was then added in PBS (pH 8.0) with 0.225% BSA (30,000 cpm/0.1 ml), and a further overnight incubation was implemented. The tubes were then aspirated, washed, and bound radioactivity counted in a Beckman model 300 biogamma counter (Fullerton, CA). A standard curve was plotted for each C3a experiment.

Measurement of C3a in aqueous humor: Approximately 98% of 125I-C3 is precipitated from normal human serum or plasma in 10 N HCl. 1 Under these conditions, 125I-C3a is completely soluble. Since anti-C3a reacts with native C3 as well as C3a in the radioimmunoassay, 0.1 ml of aqueous humor plus an equal volume of 0.9% NaCl were added to 0.02 ml 10 N HCl and incubated at room temperature for 30 sec to remove native C3. After the addition of 9 N NaOH to neutralize the HCl, the mixture was centrifuged at 4,000 rpm (Clay Adams Serofuge; Parsippany, NJ) for 15 min at room temperature. The soluble supernate containing C3a was aspirated carefully, then diluted 1:20 and 1:50, and added to the antibody-coated tubes in duplicate in 1-ml volumes for measurement in the solid phase assay. The lower limit of detection of C3a in our assay is 0.001 µg/ml.

Immunoelectrophoresis (IEP): IEP was performed by the method of Scheidigger. 7 Briefly, aqueous humor and serum frozen at −70°C were thawed, and volumes of 5 µl were added immediately to agarose gel plates (8 × 10 cm) for electrophoresis at room temperature for 90 min. After electrophoresis, antibody to β1A (C3c) and Factor B (Calbiochem-Behring; La Jolla, CA) was added to the troughs, and the plates were developed at room temperature.

Results. All six samples of normal aqueous humor obtained from patients in group 1 had no detectable C3a. We also were unable to detect Factor B or C3c in normal aqueous humor using IEP.

On the other hand, aqueous humor from all eight patients with anterior uveitis had measurable levels of C3a (Table 1). Levels of C3a did not correlate completely with the severity of the anterior chamber response. Patients 1 and 2 with rare cells and trace flare in the anterior chamber had low levels of C3a, patients 4 and 5 with 2+ cells and flare had higher levels of C3a, and patients 7 and 8 with 4+ cells and flare had the highest levels of C3a. However, patient 3 with 2+ cells and flare and patient 6 with 4+ cells and flare had relatively low concentrations of C3a. Factor B and C3c were detected by IEP in aqueous humor from patients 4–6 (3/7 of samples tested). In patients 4 and 5, Factor B was converted fully to Bb.

Discussion. In our previous studies, we attempted to characterize the complement system in both normal
and inflamed aqueous humor.\textsuperscript{3,4} We were able to demonstrate increased levels of hemolytic complement in inflamed aqueous humor. We attempted to correlate levels of hemolytic complement in aqueous humor with measurements of IgG to determine if these components were increasing proportionately or disproportionately. A greater increase of IgG relative to complement might suggest complement consumption. A comparison of the ratios of IgG to each complement component in normal and inflamed aqueous humor suggested that levels of IgG and complement increased proportionately in inflamed aqueous humor. However, complement activation and consumption may be taking place but may not be detected by our methods not only because of the wide ranges in levels of complement that we measured in inflamed aqueous humor and the wide ranges in ratios that we calculated, but also because the anterior chamber is a dynamic compartment where rapid replenishment of aqueous complement may take place from dilated, inflamed uveal vessels. Others have shown that conventional measurements of overall complement levels may be too insensitive to detect subtle but meaningful variations in complement activation.\textsuperscript{1} The detection and quantitation of cleavage products of complement is a direct assessment of activated complement\textsuperscript{1} and, thus, a major advantage over measurements of overall complement levels and comparisons with IgG.

C3a was measured by radioimmunoassay in aqueous humor from all eyes with anterior uveitis but could not be detected in normal aqueous humor. Factor B or its conversion product and C3c were found in 3/7 samples of inflamed aqueous humor but could not be detected in normal aqueous humor.

Radioimmunoassay of C3a is an extremely sensitive technique that permits quantitation of C3a in ng/ml. It is particularly suited for studies of aqueous humor because of the small volumes that are available from any one patient for analysis. These small volumes preclude the measurement of multiple factors. We quantitated C3a rather than C5a because C5a binds to a much greater degree to neutrophils and monocytes so that it is more difficult to measure and correlate with complement activation. Formation of C3a results from activation of either the classical or alternative pathways or possibly also from spontaneous breakdown of complement in the anterior chamber. Finding that Factor B was fully converted in the aqueous humor of patients 4 and 5 suggests alternative pathway activation of complement in these two cases. Regardless of how complement was activated, activated complement fragments are present in the aqueous humor of eyes with anterior uveitis and may help mediate the inflammatory process. Some of the functions of C3a include smooth muscle contraction, increased vascular permeability, and release of histamine from mast cells and lysosomal proteases from neutrophils.\textsuperscript{8}

\textbf{Key words:} complement, activated complement, aqueous humor

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